THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 25, Issue of June 23, pp. 19422–19427, 2000
Printed in U.S.A.

A Novel Binding Factor Facilitates Nuclear Translocation and Transcriptional Activation Function of the Pituitary Tumor-transforming Gene Product*

Received for publication, December 20, 1999, and in revised form, April 6, 2000
Published, JBC Papers in Press, April 25, 2000, DOI 10.1074/jbc.M910105199

Wenwen Chien and Lin Pei‡

From the Division of Endocrinology and Metabolism, Cedars-Sinai Research Institute, UCLA School of Medicine, Los Angeles, California 90048

Pituitary tumor-transforming gene (*PTTG*) is a recently characterized oncogene whose expression product contains a transcriptional activation domain at the C terminus. To understand the mechanisms involved in PTTG biological functions, we used yeast two-hybrid screening to identify proteins that interact with PTTG. This study reports the isolation and characterization of a novel PTTG-binding factor (PBF). PBF contains an open reading frame of 179 amino acids with a predicted molecular mass of 22 kDa. In Northern blot analyses, PBF mRNA was ubiquitously expressed in human tissues. Glutathione *S*-transferase pull-down and co-immunoprecipitation assays demonstrate that PBF interacts specifically with PTTG under both *in vitro* and *in vivo* conditions. The PTTG binding domain in PBF was located within the C-terminal 30-amino acid region that contain a nuclear localization signal. Immunofluorescence and subcellular fractionation studies showed that PTTG is predominantly expressed in the cytoplasm with partial nuclear localization, whereas PBF is localized both in the cytoplasm and the nucleus. The interaction between PBF and PTTG facilitated PTTG translocation from the cytoplasm to the nucleus. Furthermore, PBF is required for transcriptional activation of basic fibroblast growth factor by PTTG. In summary, we have characterized a novel PTTG-binding protein that facilitates PTTG nuclear translocation and potentiates its transcriptional activation function.

Pituitary tumor-transforming gene (*PTTG*)[1] was isolated by its differential mRNA expression in rat pituitary tumor cells (1). Overexpression of *PTTG* induces cell transformation and generates tumors in nude mice (1). Several human *PTTG* homologues have since been cloned (2–5). High level expression of PTTG mRNA in multiple types of tumors as well as in carcinoma cell lines (2–7) suggests that PTTG may be involved in tumorigenesis of many tissues in addition to the pituitary. In normal adult tissues, PTTG mRNA expression is restricted to a small number of tissues, including testis, thymus, and placenta (1–3).

The mechanisms involved in PTTG biological function are largely unknown. To elucidate the biological function of PTTG, we have used a yeast two-hybrid system to identify proteins that associate with PTTG (8). This report shows that the ribosomal protein S10 and a novel human homologue of the bacterial heat-shock protein, DnaJ (HSJ2), interact specifically with PTTG under both *in vitro* and *in vivo* conditions (8). Association of PTTG with these proteins indicates that PTTG may link to the ribosome and is involved in the regulation of translation (8). In addition, during the rat spermatogenic cycle, PTTG mRNA is expressed stage specifically in only spermatocytes and spermatids, suggesting that PTTG may play a role in rat spermatogenesis (8). The C-terminal portion of human PTTG was shown to function as a transcriptional activator when fused to a heterologous DNA binding domain (2). In NIH3T3 cells that overexpress PTTG, increased expression of bFGF mRNA and protein was observed (3). A recent study demonstrates that PTTG is a sister chromatid separation inhibitor, and degradation of PTTG is required for proper sister chromatid separation during the cell cycle (9). These observations suggest that PTTG is a multifunctional protein that can exert its effect both in the cytoplasm and the nucleus.

We now report the cloning and the characterization of a novel protein encoding a PTTG-binding factor, PBF. We have used both *in vitro* binding and immunoprecipitation assays to demonstrate that PBF interacts specifically with PTTG. With deletion analyses, we have located the regions that are required for PBF and PTTG binding to the C terminus of both proteins. We have determined the subcellular localization of PTTG and PBF and showed that co-expression of PBF increased PTTG nuclear staining. Furthermore, we have shown that PBF is required for activation of bFGF transcription by PTTG.

MATERIALS AND METHODS

Cell Lines and Transfection—COS-7 cells were cultured in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum. Transient transfections were performed using the calcium phosphate precipitation method as described previously (10).

Dot Blot and Northern Blot Analyses—Human RNA master blot and multiple tissue Northern blots were purchased from CLONTECH, and the blots were probed with human β-actin or PBF cDNA probe according to manufacturer's instructions.

Plasmids—The full-length cDNA encoding PBF with the hemagglutinin (HA) epitope from pACT2 was cloned into the *Bam*HI site of the eukaryotic expression vector pBK-CMV (Stratagene) to obtain HA-

* This work was supported by National Institutes of Health Grants DK-02346 and DK-56608 (to L. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF149785.

‡ To whom correspondence should be addressed: Division of Endocrinology and Metabolism, Cedars-Sinai Medical Center, 8700 Beverly Blvd., D2019, Los Angeles, CA 90048. Tel.: 310-423-7682; Fax: 815-352-6253; E-mail: pei@cshs.org.

[1] The abbreviations used are: PTTG, pituitary tumor-transforming gene; PBF, PTTG-binding factor; bFGF, basic fibroblast growth factor; HA, hemagglutinin; NLS, nuclear localization signal; GFP, green fluorescent protein; bp, base pairs; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; hnRNP, heterogenous nuclear ribonucleoprotein; EST, expressed sequence tag; STAT, signal transducers and activators of transcription; HCF, host cell factor.

tagged PBF (HA·PBF). The ExSite polymerase chain reaction-based site-directed mutagenesis kit (Stratagene) was used to construct the PBF nuclear localization signal (NLS) deletion mutant (HA·PBFΔNLS) in which the putative NLS sequence (amino acids 149–166) was deleted. The primers used were primer 1, 5′-TGTTTTAAAGAAGAAAACC-CGTATGCTA-3′ and primer 2, 5′-CCTCCTGCCGTATCCGCCTCTCC-T-3′.

The PBF N-terminal and C-terminal deletion mutants (N45, N90, N148, and C30) were generated from pACT2-PBF-wt using the ExSite polymerase chain reaction-based site-directed mutagenesis kit following the manufacturer's instructions (Stratagene). PTTG deletion mutants (mut(1–5)) were described previously (8).

The PTTG-green fluorescent fusion protein was obtained by subcloning the coding region of PTTG into the *Bgl*II and *Hin*dIII sites of pEGFP-C1 (CLONTECH) to obtain GFP·PTTG. The plasmid pLuc-bFGF containing the *bFGF* promoter (−1,058/+54 bp) was fused upstream of the luciferase reporter gene (11).

In Vitro Transcription/Translation of PBF—PBF cDNA was subcloned into the pBK-CMV vector (Stratagene) and used as the DNA template for the production of PBF protein. The *in vitro* transcription/translation reactions were performed using the TNT®-coupled reticulocyte lysate system with T3 RNA polymerase and biotinylated lysine-tRNA (Promega). The reactions were carried out according to manufacturer's protocols.

Glutathione S-Transferase Fusion Protein Pull-down Assays—The PTTG-GST fusion protein construct was described previously (8). Expression of the fusion protein was induced with 0.5 mM isopropyl-β-thiogalactopyranoside at 37 °C for 90 min. Cells were pelleted, resuspended in sonication buffer (150 mM KCl, 40 mM HEPES, pH 7.9, 0.5 mM EDTA, 5 mM $MgCl_2$, 10 mM dithiothreitol, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin), and lysed by sonication. The bacterial cell lysate containing GST fusion proteins was incubated at room temperature for 30 min with glutathione-Sepharose-4B beads (Amersham Pharmacia Biotech). The beads were washed three times with the sonication buffer and incubated with 20 μl of *in vitro* translated PBF at 4 °C for 2 h. The beads were then washed extensively, boiled in 2× SDS-loading buffer, and loaded onto a 10% SDS-polyacrylamide gel. After electroblotting, the blot was incubated with streptavidin-horseradish peroxidase, washed, and detected by chemiluminescence.

Immunoprecipitation—Transfected cells were lysed with 50 mM Tris, pH 7.6, 5 mM EDTA, 300 mM NaCl, 1 mM dithiothreitol, and 0.1% Nonidet P-40 in the presence of the protease inhibitors, 0.2 mM phenylmethylsulfonyl fluoride and 1 μg/ml each of leupeptin, pepstatin, and aprotinin. The cell lysate was incubated with anti-X-press antibody (Invitrogen) at 4 °C for 2 h. Immunoprecipitated complexes were bound to protein A/G-Sepharose at 4 °C. The beads were washed four times in lysis buffer, and then proteins were eluted in sample buffer and resolved on 10% SDS-PAGE. After electroblotting, the membranes were incubated with anti-HA antibody and visualized by ECL.

Yeast Two-hybrid Interaction and Colony Lift Filter Assays—These assays were performed as described previously (8).

Fluorescence Microscopy—Cells transfected with GFP·PTTG only were fixed 24–48 h post-transfection with 2% neutral buffered formaldehyde (2% formaldehyde, 20 mM $NaPO_4$, pH 7.4) in Hanks' balanced salt solution for 15 min at 37 °C, washed with phosphate-buffered saline three times, and examined under a fluorescent microscope. Cells transfected with HA·PBF or HA·PBFΔNLS, or cotransfected with GFP·PTTG and HA·PBF or GFP·PTTG and HA·PBFΔNLS were fixed and blocked with 1% fetal calf serum in phosphate-buffered saline. Cells were then incubated at 37 °C with 1:100 anti-HA antibody (Roche Molecular Biochemicals) for 1 h and with 1:10 anti-mouse IG-rhodamine (Roche Molecular Biochemicals) for 1 h at 37 °C, with three phosphate-buffered saline washes after each incubation. Slides were examined with fluorescence microscopy. The fluorescence data are based on multiple transfection experiments.

Subcellular Fractionation—The nuclear and cytosol fractions were prepared using the method described by Dignam *et al.* (12) from COS-7 cells transfected with PTTG, PBF, or both expression plasmids. The samples were resolved on 10% SDS-PAGE. After electroblotting, the membranes were incubated with various antibodies and analyzed by ECL. The antibodies include a monoclonal antibody against hnRNP (diluted 1:1000) and a polyclonal antibody against Raf1 at a dilution of 1:200 (Santa Cruz Biotechnology).

Luciferase Assays—Luciferase assays were performed as described previously (6).

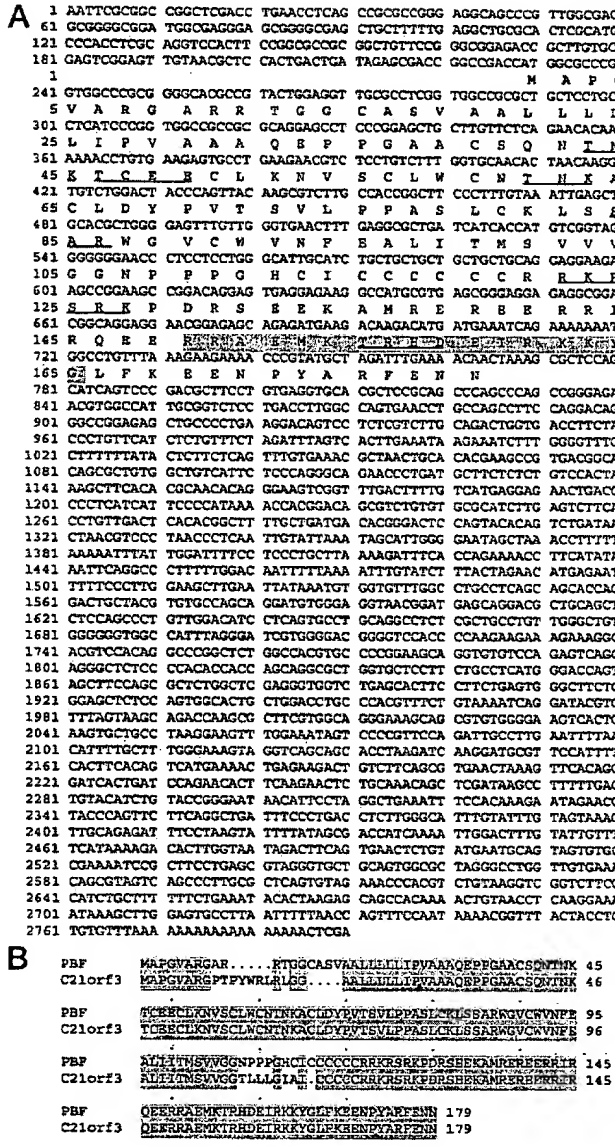

FIG. 1. **Sequence alignment and structural features of PBF.** *A*, PBF nucleotide and amino acid sequence. The deduced amino acid sequence of PBF is shown below the cDNA sequence. The nucleotide and amino acid numbers are labeled to the *left* of each *lane*. Phosphorylation sites for protein kinase C, cAMP- and cGMP-dependent protein kinases, and casein kinase II are *underlined*. The potential nuclear localization signal is *shaded*. *B*, amino acid sequence alignment of PBF and C21orf3. A BESTFIT comparison is shown. The identical amino acid residues between the two proteins are *shaded*.

RESULTS

Cloning of a cDNA Encoding PBF—Using the yeast two-hybrid screen with rat PTTG as bait, several positive clones were isolated from a human testis cDNA library (8). One cDNA of 2790 bp has a canonical initiation codon at nucleotide 229 and a 3′-untranslated region of 2025 bp (Fig. 1*A*). This cDNA, designated as PBF, contains an open reading frame of 179 amino acids (Fig. 1*A*) with a predicted molecular mass of 22 kDa and a pI of 10.57.

Homology searches against the EBI and GenBank™ data bases reveal that the PBF amino acid sequence has 92% identity to a previously characterized cDNA termed C21orf3 (13) (Fig. 1*B*). The *C21orf3* gene was mapped to chromosome 21q22.3 where several hereditary disorders have been linked (14, 15). Nucleotide sequence comparisons against the expressed sequence tag (EST) database identified more than 100 human ESTs derived from lung, uterus, heart, testis, bone

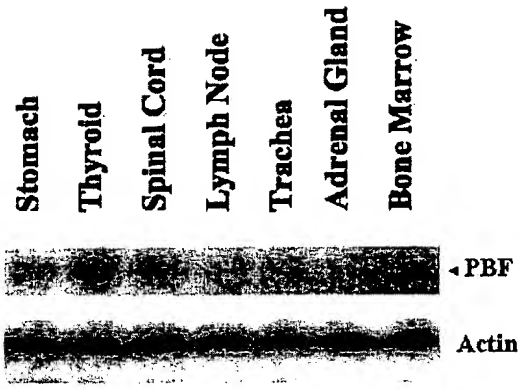

FIG. 2. **Tissue distribution and transcript size of PBF mRNA.** Poly(A$^+$) RNA from indicated human adult tissues were probed with a PBF cDNA probe. The 2.8-kilobase PBF transcript is indicated by an *arrow*. The blot was also probed with human actin cDNA to ensure equal loading of RNA on each *lane*.

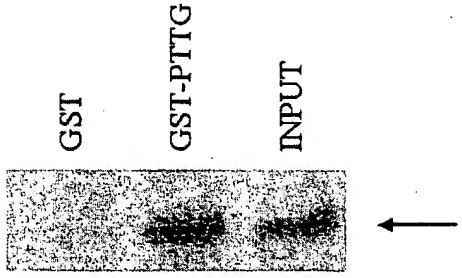

FIG. 3. ***In vitro* interaction between PBF and PTTG.** *In vitro* transcribed and translated PBF (*input*) was incubated with glutathione-Sepharose-bound GST (*lane 1*) or GST-PTTG fusion protein (*lane 2*). The bound proteins were resolved on 10% SDS-PAGE, blotted, and visualized by ECL.

A PBF Gal AD Fusion

wt	1	179
N45	46	179
N90	91	179
N148	149	179
C30	1	148

B PBF/PTTG Interaction

GBD	GAD	Colony	Filter
PTTG wt	PBF wt	+	Blue
PTTG wt	PBF N45	+	Blue
PTTG wt	PBF N90	+	Blue
PTTG wt	PBF N149	+	Blue
PTTG wt	PBF C30	-	N/A
PTTG mut4	PBF wt	+	Blue
PTTG mut5	PBF wt	-	N/A

FIG. 4. **Identification of PBF/PTTG interactive domains.** *A*, schematic diagram of Gal4 activation domain (*Gal AD*) and PBF deletion fusion constructs. *B*, interaction between various deletion mutants of PBF and PTTG in the yeast two-hybrid system. Yeast cells (GC1945) were cotransformed with the indicated pair of fusion constructs. Colonies grown on the selective medium were tested for β-galactosidase activity by colony lift filter assay. *wt*, wild type; *GAD*, Gal4 activation domain; *GBD*, Gal4 binding domain.

marrow, pancreas, spleen, melanocytes, and neurons. Identity matches were also found with human ESTs corresponding to cDNAs overexpressed in colon carcinomas, Wilms' tumor, and parathyroid tumors. Several mouse ESTs showed more than 80% identity to PBF and probably represent the murine homologue of PBF.

Analysis of protein sequence using the PROSITE data base of protein sites and patterns revealed putative phosphorylation sites for cyclic AMP- and GMP-dependent protein kinase, protein kinase C, and casein kinase II (Fig. 1*A*). Five potential glycosylation sites for *N*-linked and *O*-linked oligosaccharides were found. Prediction of sorting signals and cellular localization for PBF was performed using the PSORT program. A cleavable N-terminal signal was identified between amino acids 31 and 32. A potential nuclear localization signal sequence was identified at the C terminus (Fig. 1*A*).

Tissue Distribution of Human PBF—The tissue distribution of PBF mRNA was determined using a human RNA master blot. The results show that PBF mRNA is ubiquitously expressed in all the tissues analyzed, with the highest level of expression detected in placenta (data not shown). To determine the transcript size of the PBF mRNA, Northern blot analysis was performed on selected tissues. As shown in Fig. 2, a unique transcript of 2.8 kilobases was observed (Fig. 2, *upper panel*). The transcript size on the Northern blot suggests that the PBF cDNA clone (2790 bp) is likely to be full length. The membrane was also probed with the actin control to confirm mRNA integrity as well as loading efficiency (Fig. 2, *lower panel*).

In Vitro Interaction between PBF and PTTG—To test the binding specificity of PBF to PTTG, the ability of *in vitro* transcribed and translated PBF to bind bacteria-expressed PTTG was examined. PBF was retained on Sepharose beads only in the presence of GST-PTTG (Fig. 3, *lane 2*), whereas no protein was retained when GST alone was added (Fig. 3, *lane 1*). These results demonstrate that PBF specifically binds PTTG *in vitro*.

PBF and PTTG Interact via C-terminal Regions of Both Proteins—To determine the regions of PBF involved in interaction with PTTG, PBF deletion mutants fused to the yeast Gal4 activation domain vector were constructed (Fig. 4*A*). These fusion plasmids were used to cotransform yeast with the hybrid plasmid containing PTTG and the Gal4 DNA binding domain. The interaction between PBF deletion mutants and PTTG was monitored by the expression of *HIS3* and *lacZ* reporter genes, which were monitored by growth on histidine-deficient medium and production of β-galactosidase, respectively. Deletion of 148 amino acids from the N terminus (N45, N90, and N148) had no effect on PBF/PTTG interaction (Fig. 4*B*). However, deletion of the 30 amino acids from the C terminus (C30) abolished interaction between PBF and PTTG (Fig. 4*B*). These results suggest that the 30 amino acids that include the nuclear localization signal in the C terminus of PBF are necessary and sufficient for PBF binding to PTTG.

To determine the region of PTTG involved in interaction with PBF, PTTG N-terminal deletion mutants constructed in the Gal4 DNA binding domain vector (8) and PBF constructed in the Gal4 activation domain vector were used in the similar yeast two-hybrid assays. No change in the interaction between PBF and PTTG was detected when the N-terminal 123-amino acid deletion mutant (PTTG mut4) was used (Fig. 4*B*). The PBF/PTTG interaction was completely abrogated only when the N-terminal 154 amino acids were deleted (PTTG mut5). These results suggest that the region between amino acids 123 and 154 of PTTG is essential for interaction with PBF.

Interaction of PBF and PTTG in Vivo—To determine

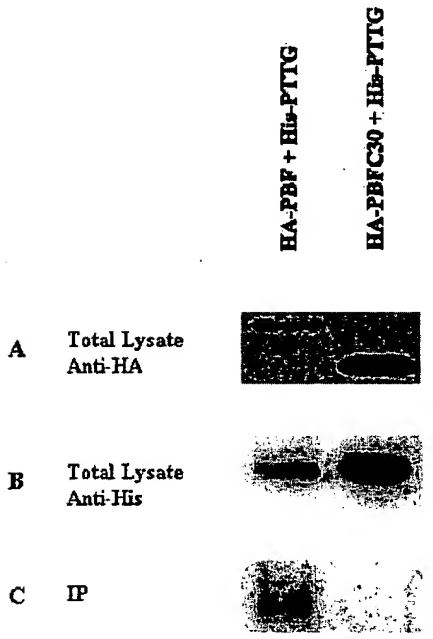

FIG. 5. ***In vivo* association of PBF and PTTG.** COS-7 cells were transiently transfected with the indicated plasmids. *A* and *B*, Western blot analysis using anti-HA (*A*) and anti-His (*B*) monoclonal antibodies. *C*, immunoprecipitation (*IP*) of cell lysates prepared from transfectants with anti-His monoclonal antibody. The immunocomplexes were separated on SDS-PAGE, blotted, and probed with anti-HA monoclonal antibody.

whether PBF and PTTG associate with each other in mammalian cells, we transiently cotransfected COS-7 cells with HA-tagged PBF (wild type and C-terminal 30-amino acid deletion mutant) and His-tagged PTTG expression plasmids. Total lysates were subjected to Western blot analyses to confirm the expression of individual protein in the transfected cells (Fig. 5, *A* and *B*). The lysates were immunoprecipitated with anti-His antibody, and the bound protein was detected by Western blot analysis using anti-HA antibody. As shown in Fig. 5, anti-HA antibody detected a band in lysates from cells cotransfected with the wild type PBF and PTTG expression plasmids, whereas no band was detected in lysates from cells cotransfected with PTTG and the C-terminal deletion mutant of PBF (Fig. 5, *C*). This is consistent with the results from the yeast two-hybrid assays above in which the C-terminal 30-amino acid domain was shown to be required for PBF binding to PTTG. The results suggest that PBF interacts specifically with PTTG in mammalian cells via the 30 amino acids at the C terminus.

Subcellular Localization of PBF—PBF contains a bipartite nuclear localization signal between amino acids 149 and 166 at the C terminus. To test whether this potential NLS has any effect on PBF subcellular localization, the HA-tagged PBF constructs containing either a full-length PBF (HA·PBF) or a deletion mutant lacking the NLS sequence (HA·PBFΔNLS) were transfected into COS-7 cells. As shown in Fig. 6*A*, HA·PBF was mainly expressed in the nucleus, but there was also significant expression in the cytoplasm. In contrast, the NLS deletion mutant, HA·PBFΔNLS, yielded a predominantly perinuclear and cytoplasmic staining pattern (Fig. 6*B*). These results suggest that NLS is required for PBF localization to the nucleus.

PBF Facilitates PTTG Nuclear Translocation—To characterize the biological significance of the interaction between PBF and PTTG, both proteins were transfected into COS-7 cells, and the subcellular localization was examined. Initially COS-7 cells were transiently transfected with GFP·PTTG only. Expression of GFP·PTTG was predominantly observed in the cytoplasm (Fig. 7*A*) with partial nuclear localization. When COS-7 cells

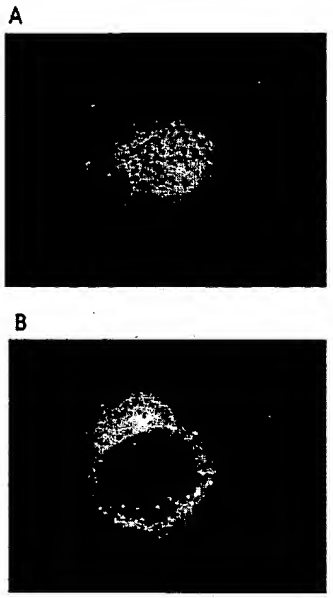

FIG. 6. **Subcellular localization of PBF.** COS-7 cells were transfected with HA·PBF (*A*) or HA·PBFΔNLS (*B*). 24–48 h after transfection cells were fixed. HA-tagged proteins were detected with anti-HA monoclonal antibody and a rhodamine-conjugated secondary antibody.

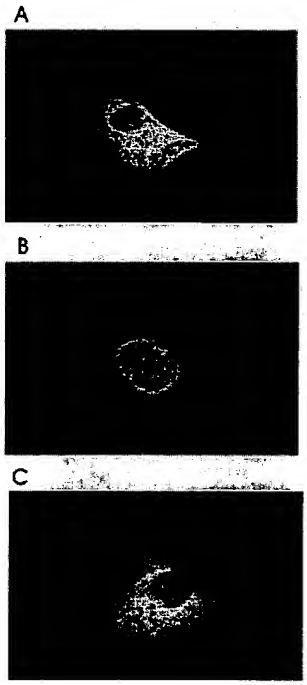

FIG. 7. **Nuclear translocation of PTTG by PBF.** COS-7 cells were transfected with GFP·PTTG (*A*), GFP·PTTG and HA·PBF (*B*), or GFP·PTTG and HA·PBFΔNLS (*C*). Twenty-four hours after transfection, cells were fixed. GFP·PTTG was detected by green fluorescence.

were cotransfected with both GFP·PTTG and HA·PBF, most of the GFP·PTTG expression was now localized to the nucleus (Fig. 7*B*). Our deletion studies using yeast two-hybrid assays showed that the C-terminal 30-amino acid domain including the nuclear localization signal is required for PBF binding to PTTG. We then tested whether the NLS deletion construct of PBF was still able to promote PTTG nuclear accumulation. As shown in Fig. 7*C*, in cells cotransfected with GFP·PTTG and HA·PBFΔNLS, the expression of GFP·PTTG remained mainly in the cytoplasm, indicating that PBF lacking the NLS can no longer bring PTTG into the nucleus.

The relative nuclear *versus* cytoplasmic distribution of PBF

and PTTG was also examined by subcellular fractionation. Nuclear or cytoplasmic fractions were prepared from transfected cells. The purity of the fractions was verified by probing the membrane with the nuclear (anti-hnRNP) or cytoplasmic (anti-Raf1) protein specific antibody. As shown in Fig. 8, in cells transfected with PTTG alone the majority of the PTTG was detected in the cytoplasm. In contrast, in cells cotransfected with PBF and PTTG expression plasmids, there was a dramatic increase in the immune activity of PTTG in the nuclear fraction (Fig. 8). Quantitation of the immunoblot showed that the amount of PTTG present in the nucleus increased from less than 15% to more than 70% after coexpression with PBF (not shown). These results indicate that the interaction between PBF and PTTG facilitates PTTG translocation from the cytoplasm to the nucleus, and the nuclear localization signal of PBF is required for PTTG nuclear translocation.

PBF Is Required for PTTG Activation of bFGF Transcription—To further explore the functional implication of PBF/PTTG interaction, we tested whether PBF affects the function of PTTG as a transcriptional activator. Previously it had been demonstrated that bFGF expression was induced in NIH3T3 cells that overexpress PTTG (3). To test whether PTTG was capable of activating *bFGF* transcription, COS-7 cells were transiently transfected with *bFGF* promoter linked to luciferase together with the PTTG expression plasmid. As shown in Fig. 9, transfection of PTTG expression vector alone had little effect on luciferase activity of the *bFGF* reporter gene. Similarly, transfection of PBF expression vector alone did not change the reporter gene activity (Fig. 9). However, when PTTG and PBF were coexpressed, the luciferase activity of the reporter gene was induced more than 3-fold. These results suggest that PBF is required for PTTG activation of *bFGF* transcription.

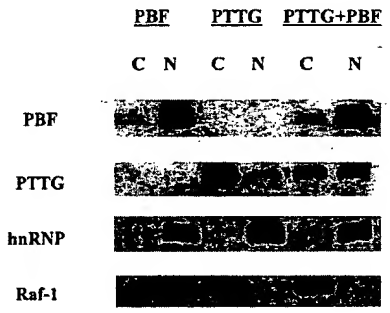

FIG. 8. **Subcellular localization of PTTG and PBF.** The nuclear and cytosol fractions were prepared as described under "Materials and Methods." The purity of the fractions was verified using anti-Raf1 (cytosol) and anti-hnRNP (nuclear) antibodies. GFP·PTTG was detected by anti-GFP antibody. HA·PBF protein was detected with anti-HA antibody. The plasmids used for transfection are indicated on the *top* of the *panels*. *C*, cytosol; *N*, nuclear fraction.

DISCUSSION

In this study, we report the isolation and characterization of a novel PTTG-binding factor. PBF shares its highest sequence homology with a previously isolated cDNA, C21orf3 (13). Although the *C21orf3* gene has been mapped to chromosome 21q22.3, where several hereditary disorders have been linked (14, 15), the functions of the *C21orf3* gene product have not been characterized. The PBF mRNA tissue distribution pattern is similar to that of C21orf3, which is also ubiquitously expressed.

We have used biochemical and immunological methods to show that PBF can form a complex with PTTG under both *in vitro* and *in vivo* conditions and that this interaction requires the C-terminal portion of both proteins. The sequence required for PTTG to interact with PBF was located within the C-terminal 75-amino acid domain. This result agrees with the previous study in which the interactive domain of PTTG with S10 and HSJ2 proteins was mapped to the same region (8). Several motifs that are known to mediate protein-protein interactions are present in this region, including a leucine-zipper motif (16) between amino acids 169 and 190, as well as two putative SH3 (17) domains at amino acids 169 and 176. The functional significance of these motifs in mediating interaction of PTTG with other proteins is currently being investigated. Our results also showed that the C-terminal 30 amino acids of PBF are both necessary and sufficient to mediate its interaction with PTTG.

PBF contains a bipartite NLS between amino acids 149 and 166. Proteins entering the nucleus are directed by NLS sequences that are present in nuclear proteins (18). The NLS sequences are recognized by specific cytosolic proteins that help to direct the nuclear protein to the nuclear pore complex and its translocation through the nuclear envelope (19–21). The NLS is generally either a short basic region of 4–8 amino acids or a bipartite basic sequence separated by 4–15 amino acids (22–24). Our results show that PBF is predominantly localized to the nucleus and that deletion of the NLS sequence abolished PBF nuclear localization. This indicates that the NLS sequence is required for PBF nuclear localization as well as interaction with PTTG.

PTTG does not contain a consensus NLS sequence, and our indirect immunofluorescence and subcellular fractionation studies show that PTTG exhibits a predominantly cytoplasmic localization. This result is in agreement with results from a previous study indicating that human PTTG is mainly expressed in the cytoplasm (2). However, coexpression of both PBF and PTTG in the same cells resulted in translocation of PTTG from the cytoplasm to the nucleus. Our studies indicate that nuclear translocation of PTTG requires the presence of the NLS of PBF because coexpression of a PBF mutant that lacks its NLS was unable to bind PTTG and failed to promote PTTG

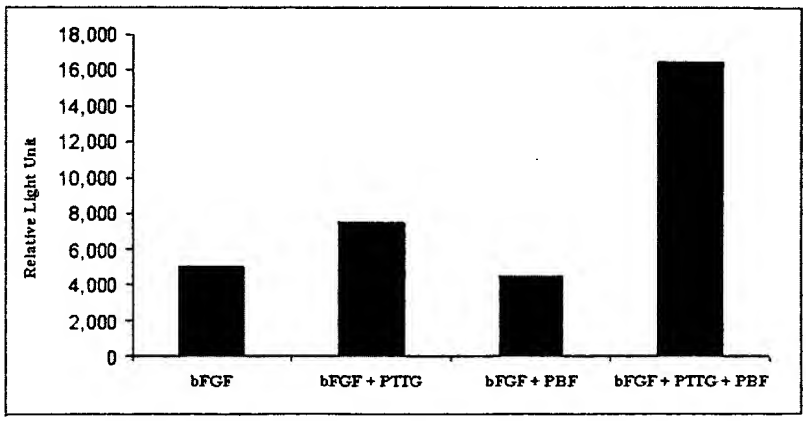

FIG. 9. **PBF is required for transcriptional activation of bFGF by PTTG.** COS-7 cells were transiently cotransfected with pCMV-PTTG or pCMV-PBF, either alone or together, and a reporter plasmid containing −1054 bp of the bFGF promoter sequence fused to the reporter gene luciferase construct (bFGF-LUC). Forty-eight hours after transfection, cell extracts were assayed for luciferase activity that is represented as *relative light units*. *Bars* indicate the mean of three independent experiments.

nuclear accumulation. These results suggest that one of the functions of PBF is to bind PTTG and direct it into the nucleus. This function of PBF is reminiscent of the function of HCF, a cellular factor involved in herpes simplex virus immediate-early gene induction (25, 26). HCF is part of a multicomponent protein complex containing VP16 and Oct-1 (25, 26). A recent study showed that nuclear trafficking of VP16 is HCF-dependent (27). Like PTTG, VP16 itself does not contain a consensus NLS and is localized predominantly in the cytoplasm. It was shown that coexpression with HCF resulted in VP16 nuclear translocation and that the NLS within the C terminus of HCF was required for this function (27).

It has been shown that the C-terminal portion of human PTTG has transcriptional activation activity when fused to a heterologous DNA binding domain (2). For PTTG to function as a transcriptional activator, the presence of PTTG in the nucleus is required. Our results show that transcriptional activation of the *bFGF* gene requires the presence of both PTTG and PBF. These results suggest that PBF potentiates the PTTG transactivation function by facilitating its nuclear translocation. Another common mechanism that affects the subcellular distribution and functions of a protein is phosphorylation. One such example is the family of transcription factors called STAT proteins (signal transducers and activators of transcription). STAT proteins are involved in the initiation of gene expression by many cytokines and cell growth factors. Upon activation by tyrosine phosphorylation through the cytoplasmic domain of stimulated receptors, the phosphorylated STAT proteins dimerize and are translocated to the nucleus for transcriptional activation by binding to specific recognition sites (28). There are several potential phosphorylation sites for different kinases on both PBF and PTTG, and the effects of phosphorylation on the subcellular distribution and functions of these proteins are currently being investigated.

In summary, this work provides biochemical and functional evidence that PBF binds PTTG directly and is able to promote PTTG nuclear translocation and its transcriptional activation activity. Interaction between PBF and PTTG and the subsequent nuclear translocation of PTTG suggest a potential mechanism by which PTTG might function as a transcriptional activator.

Acknowledgments—We thank Dr. Peter A. Cattini and Dr. G. Dreyfuss for providing the bFGF-luc plasmid and anti-hnRNP antibody, respectively.

REFERENCES

1. Pei, L., and Melmed, S. (1997) *Mol. Endocrinol.* **11,** 433–441
2. Dominguez, A., Ramos-Morales, F., Romero, F., Rios, R. M., Dreyfus, F., Tortolero, M., and Pintor-Toro, J. A. (1998) *Oncogene* **17,** 2187–2193
3. Zhang, X., Horwitz, G. A., Prezant, T. R., Valentinin, A., Nakashima, M., Bronstein, M. D., and Melmed, S. (1999) *Mol. Endocrinol.* **13,** 156–166
4. Lee, I. A., Seong, C., and Choe, I. S. (1999) *Biochem. Mol. Biol. Int.* **47,** 891–897
5. Kakar, S. S. (1999) *Gene (Amst.)* **240,** 317–324
6. Pei, L. (1998) *J. Biol. Chem.* **273,** 5219–5225
7. Saez, C., Japon, M. A., Ramos-Morales, F., Romero, F., Segura, D. I., Tortolero, M., and Pintor-Toro, J. A. (1999) *Oncogene* **18,** 5473–5476
8. Pei, L. (1999) *J. Biol. Chem.* **274,** 3151–3158
9. Zou, H., McGarry, T. J., Bernal, T., and Kirschner, M. W. (1999) *Science* **285,** 418–422
10. Graham, F. L., and van der Eb, A. J. (1973) *Virology* **52,** 456–467
11. Pasumarthi, K. B. S., Jin, Y., and Cattini, P. A. (1997) *J. Neurochem.* **68,** 898–908
12. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489
13. Yaspo, M. L., Aaltonen, J., Horelli-Kuitunen, N., Peltonen, L., and Lehrach, H. (1998) *Genomics* **49,** 133–136
14. Aita, U. M., Liu, J., Knowles, J. A., Terwilliger, J. D., Baltazar, R., and Grunna, A. (1999) *Am. J. Hum. Genet.* **64,** 210–217
15. Sinet, P. M., Theophile, D., Rahmani, Z., Chettouh, Z., Blouin, J. L., Prieur, M., Noel, B., and Delebar, J. M. (1994) *Biomed. Pharmacother.* **48,** 247–252
16. Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988) *Science* **240,** 1759–1764
17. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991) *Science* **252,** 668–674
18. Dingwall, C., and Laskey, R. A. (1991) *Trends Biochem. Sci.* **16,** 478–481
19. Corbett, A. H., and Silver, P. A. (1997) *Microbiol. Mol. Biol. Rev.* **61,** 193–211
20. Pemberton, L. F., Blobel, G., and Rosenblum, J. S. (1998) *Curr. Opin. Cell Biol.* **10,** 392–399
21. Adams, S. A., and Gerace, L. (1991) *Cell* **66,** 837–847
22. Kalderon, D., Robers, B. L., Richardson, W. D., and Smith, A. E. (1984) *Cell* **39,** 499–509
23. Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991) *Cell* **64,** 615–623
24. Nigg, E. A. (1997) *Nature* **386,** 779–787
25. O'Hare, P. (1993) *Semin. Virol.* **4,** 145–155
26. Wilson, A. C., Cleary, M. A., Lai, J. S., LaMarco, K., Peterson, M. G., and Herr, W. (1993) *Cold Spring Harbor Symp. Quant. Biol.* **58,** 167–178
27. Boissiere, S. L., Hughes, T., and O'Hare, P. (1999) *EMBO J.* **18,** 480–489
28. Koster, M., and Hauser, H. (1999) *Eur. J. Biochem.* **260,** 137–144

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☑ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.